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Date of Deposit: April 15, 2005

Attorney Docket No: 21486-027DIV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Hopkins et al		
SERIAL NUMBER:	10/722,279	EXAMINER:	Javier G. Blanco
FILING DATE:	November 24, 2003	ART UNIT:	3738
FOR:	CARDIAC VALVE REPLACEMENT		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. § 1.131

We, Richard A. Hopkins and Dianne Hoffman-Kim, declare:

1. We are the inventors of the invention claimed in the above-referenced patent application.
2. The invention was completed in the United States prior to November 22, 1999, the effective filing date of Wolfinbarger, Jr. (U.S. Patent No.6,432,712) under 35 U.S.C. § 102(e). The Wolfinbarger, Jr. reference was cited by the Office in the October 15, 2004 Office Action.
3. The evidence provided in this declaration demonstrates that the invention claimed in this application was made before the November 22, 1999 date of Wolfinbarger, Jr.
4. We conceived of the invention claimed in the above-referenced patent application in the United States before the effective filing date of Wolfinbarger, Jr. Furthermore, we diligently worked to reduce our invention to practice from prior to November 22, 1999 up until April 7, 2000, at which time we constructively reduced our invention to practice by filing U.S. Provisional Patent Application No. 60/195,673. The present application is a divisional application of U.S. Patent Application No. 09/828,768 (now U.S. Patent No. 6,652,583), filed on April 9, 2001, which claims priority from U.S. Provisional Patent Application No. 60/195,673.
5. We submit herewith a copy of the invention disclosure (Exhibit A), which describes the claimed method and which we forwarded to our patent counsel prior to the November 22, 1999 effective filing date of Wolfinbarger, Jr..

Applicants: Andrew Hoffman
U.S.S.N. 09/616,483

6. Also submitted is a copy of a letter that includes our comments regarding a set of draft claims prepared by our patent counsel (Exhibit B). This letter was sent to our patent counsel on January 5, 2000.

7. Exhibit C is a copy of a draft patent application that our patent counsel prepared for our review. This draft was sent to us on January 31, 2000.

8. Exhibit D is a copy of the draft patent application that includes our comments. This marked-up draft was sent to our patent counsel on February 17, 2000.

9. We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Declarant's Signature
Full Name of Declarant: Richard A. Hopkins

Date

Declarant's Signature
Full Name of Declarant: Diane Hoffman-Kim

15 April 2005
Date

Attachments:
Exhibit A: Invention Disclosure
Exhibit B: Letter
Exhibit C: Draft Application
Exhibit D: Marked-up Draft Application

TRA 2027095v1

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(*) _____ 4/15/05
Declarant's Signature Date
Full Name of Declarant: Richard A. Hopkins

_____ _____
Declarant's Signature Date
Full Name of Declarant: Diane Hoffman-Kim

Attachments:
Exhibit A: Invention Disclosure
Exhibit B: Letter
Exhibit C: Draft Application
Exhibit D: Marked-up Draft Application

CONFIDENTIAL

Disclosure of Invention

I. Invention Title

A tissue engineered living replacement heart valve for use in children and adults

II. Inventors

Richard A. Hopkins, MD, Diane Hoffman-Kim, PhD

III. Date and place at which discovery was made

Rhode Island Hospital

IV. Names and Addresses of any other persons who participated in the invention

None

V. Contributions from Rhode Island Hospital to the invention:

Funds: Dr. Hopkins contractual fund?
Collis philanthropic fund?

Personnel: Salaries?

Space: Aldrich 722, 723

Facilities: None

VI. Other sources of support from sponsors (if applicable):

None

VII. Invention disclosed on a particular date to particular parties

Lifenet, AMDI (both under confidentiality agreements)
Rhode Island Foundation, The Children's Heart Foundation (both private non-profit funding agencies)

CONFIDENTIAL DRAFT

VIII. Concise description of the invention

We are disclosing a process by which to create a recellularized homograft valve for implantation, that contains cells which are genetically identical to or tolerated by the putative recipient. Significantly, this will provide a potentially permanent heart valve replacement that can grow, repair injury, and adapt to changing stress just as an original cardiac valve would be expected to do. The objective of this work is to achieve replacement of the cardiac valve with a living bioprosthesis which closely resembles and approximates the function of the native valve with regard to the following properties: mechanical, physiological, repair, remodeling and growth capabilities, cell and matrix composition, and cellular and molecular functions. Such a prosthesis would reduce the need for reoperative replacement, as it would possess perfect hemodynamic function without a need for anticoagulation or immunosuppression, with durability extending potentially to the recipient's natural lifespan.

Preferred version of the invention

Our recellularization process will ensure the presence of cells with the mechanical properties and synthetic potential necessary for ongoing reconstruction of the biochemical milieu of an initially inert protein matrix. The cells will be appropriate in their genome and phenotypic expression and will communicate appropriately with other required cell populations. Specifically, interstitial matrix cells will be predominantly myofibroblasts containing actin and myosin as well as specific secretory capabilities specific to cardiac valve leaflets. For example, there must be appropriate synthesis of type I collagen as opposed to type III or scar collagen. The extracellular matrix components of the valve replacement will initially be derived from a decellularized donor valve matrix, but will ultimately be produced by the myofibroblasts in the valve. Thus, this valve prosthesis will contain myofibroblasts and extracellular matrix in the internal layers with a single cell layer of endothelial cells on the outermost blood-contacting surfaces.

To produce a valve with this specific structure, the patient's own cells will be harvested and grown in tissue culture. The de-differentiation of the cells will be prevented through culturing in environments that are conducive to cardiac valve myofibroblast and endothelial cell phenotypes. Promotion of the myofibroblast and endothelial cell phenotypes will be through genetic manipulation and/or manipulation of the cells' environment *in vitro*. *In vitro* cellular environments to promote the proper phenotype will include co-culture of early passage endothelial cells and myofibroblasts without physical contact, to allow circulation of diffusable cell signaling factors between the two cell types. The cells will also experience pulsatile flow conditions and forces identical to those of the native valve. These hydrodynamic culture properties will directly influence the maintenance of cellular phenotype essential for the recellularization process.

General description of the invention

The tissue engineered valve replacement is comprised of cells incorporated within a molecular matrix. While the matrix will ultimately be synthesized by the valve cells, the initial matrix may be composed of synthetic (permanent or resorbable), natural or

biomodified materials. Matrices may also derive from xenografts or allografts, either decellularized or containing donor cells. The cellular component of the tissue engineered valve has a range of potential sources. An auto-seeded valve may be created by attracting the patient's own cells onto an implanted acellular valve matrix via the presence of biochemical attractant factors. The valve matrix may also be pre-seeded prior to implantation. Valve cells could include the patient's own cells (myofibroblasts and endothelial cells) biopsied from cardiac valve and/or other vascular or non-vascular structures. If donor cells can be rendered non-antigenic through masking of antigenic proteins, then allografts or xenografts may be possible. Cells may be genetically modified to promote the desired phenotypes. Stem cells may generate differentiated cells appropriate for seeding of matrices. Recent work in which myoblast satellite cells from autologous skeletal muscle were able to differentiate into cardiac myocytes suggests interesting possibilities for this area.

Assessment of a functional valve replacement will include criteria of:

1. Cell viability – We will pay close attention to cell state in light of our recent studies of apoptosis (Hilbert, et al. 1999).
2. Cell functionality:
   a. Endothelial cells: standard assays to ensure maintenance of permeability barrier.
   b. Myofibroblasts: contractile and synthetic properties
3. Matrix composition:
   a. Collagen I
   b. Elastin
   c. Glycosaminoglycans
4. Presence, amounts, and continued synthesis of all matrix components

IX. Differences from prior art

Differences from prior art include the requirement of a specific cell type, the myofibroblast, within the cellular component. Cells will be viable and functional according to current definitions. Particular attention will be paid to cell state, in light of our recent studies of apoptosis in cryopreserved homografts. Key cell functions will be maintained through prevention of de-differentiation or promotion of phenotype, through the processes of cell culture and recellularization of an acellular valve matrix. The myofibroblast cell type responds to specific signals for varying ratios of synthesis of matrix components, such as elastin, chondroitin sulfate, etc. Thus, our recellularization methods insure immune tolerance, cell to cell signaling and prevention of apoptosis.

EXHIBIT B

From: Diane Hoffman-Kim To: Fish & Richard Ingrid Beattie Date: 01/05/2000 Time: 2:55:18 PM Page 1 of 3

FACSIMILE COVER PAGE

To :	**Fish & Richard Ingrid Beattie**	**From :**	Diane Hoffman-Kim
Sent :	01/05/2000 at 2:55:18 PM	**Pages :**	3 (including Cover)
Subject :	Additions to Draft Claims		

Hi Ingrid,

Richard and I had a chance to go through the draft claims. Here are our thoughts, in note form. Let us know what you think.

RECEIVED
JAN 05 2000
INGRID A. BEATTIE

Thanks,
Diane Hoffman-Kim

3:17
RECEIVED
JAN 05 2000

Richard A. Hopkins, M.D.
Diane Hoffman-Kim, Ph.D.

CONFIDENTIAL

DRAFT

5 January 2000
Ingrid,
I'm sending some notes to supplement the Draft Claims. In most cases, I believe that what Richard and I are adding will be split into multiple claims, but I leave that to your more knowledgeable discretion.
Thanks,
Diane

Additions to Draft Claims

Claim 1
60% type I collagen
Use ratio 60:40
State total % of type I collagen.
State in terms of –fold more

Claims 1-4
Include other matrix components produced by myofibroblasts:
"Wherein said myofibroblast produces extracellular matrix components including fibronectin, elastin, and glycosaminoglycans, specifically chondroitin sulfate and hyaluronic acid"

Claims 5-6
Include other tissue sources
"Wherein said myofibroblast is derived from vascular, dermal, and other fibroblast sources and differentiated such that the critical cardiac leaflet myofibroblast phenotype is attained and maintained."
Define myofibroblast phenotype? In Claims or in body of patent?

Claim 9
Expand to include known or unknown factors, proteins, glycosaminoglycans derived from myofibroblasts and/or endothelial cells and/or the cells' conditioned medium. These factors prevent cellular differentiation away from the leaflet myofibroblast phenotype.

Claim 11
"The valve of claim 1, wherein the actual and/or functional anatomy approximates that of the native valve. Specifically, the valve leaflets contain a monolayer of endothelial cells on the external layer and myofibroblasts in the inner layers. The leaflet interstitium contains a non-homogeneous matrix of more than one layer with myofibroblasts present in all layers, with collagen fibrils oriented in more than one direction."

Claim 12
"inhibiting apoptosis of a harvested, cultured, transformed, and/or transplanted myofibroblast"

Claim 13
Replace "porcine hepatocytes" with "myofibroblasts".

Claim 15
Distinguish co-culture sequentially (i.e. separately first) vs. concomitantly.
*Note this may apply to other claims as well, such as Claim 10.

Claim 16
Rather than specifying the ratio of MF:EC, add the possibility of a third cell type, a secretory cell. As an alternative to conditioned medium, a matrix component could be produced from a genetically modified cell of non-endothelial and non-myofibroblast origin. Known or unknown signaling factors could be produced as well.
"Wherein said myofibroblast (+/- endothelial cell) is cultured in the presence of a cell genetically modified to produce signaling factors and/or extracellular matrix components."

Claim 17
"A fibroblast, genetically modified to resemble the myofibroblast phenotype with respect to contractile capability and synthetic capability, i.e. producing collagen I, fibronectin, glycosaminoglycans."

Claim 19a
Rather than "providing" the matrix, state "beginning with" the matrix.

Claim 19b
"seeding, synthetically or autologously (in vitro or in vivo), said matrix with isolated myofibroblasts +/- endothelial cells +/- other cells; one or more treated/prepared in the above manner"

Claim 19c
"culturing said myofibroblasts +/- endothelial cells +/- other cells under pulsatile flow conditions, and/or with conditioned medium and/or isolated soluble factors"

Other notes
Preferred matrices are acellular homograft, acellular xenograft, or a synthetic matrix designed to simulate these.

Should we claim specific endpoints in preparation; when the valve is ready for implantation?

Claims 7 and 13 seem to claim a process that always includes pulsatile flow. While we think pulsatile flow will be a very important part of the process, we don't want to exclude the possibility that co-culture of myofibroblasts and endothelial cells (or myofibroblast cells + endothelial cell conditioned medium) under static conditions may be enough to attain or maintain the myofibroblast phenotype.

EXHIBIT C

FISH & RICHARDSON P.C.

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January 31, 2000

By Facsimile

Diane Hoffman-Kim
Assistant Professor
Rhode Island Hospital
Aldrich 72
593 Eddy Street
Providence, RI 02903

Re: CARDIAC VALVE REPLACEMENT

Applicant: Hoffman-Kim et al.
Our Ref.: 04930-027001

Dear Diane:

Enclosed is a copy of a draft patent application. Specific questions are highlighted in bold, underlined type. Please call me with your comments.

Very truly yours,

IABeattie

Ingrid A. Beattie, Ph.D., J.D.

IAB/lar

cc/enc: Ms. Peggy McGill
Dr. Richard Hopkins

20022597.doc

CARDIAC VALVE REPLACEMENT

STATEMENT AS TO FEDERALLY-SPONSORED RESEARCH

BACKGROUND OF THE INVENTION

The invention relates to cardiac valve replacement.

Cardiac valve diseases are prevalent clinical problems, usually requiring prosthetic replacement. Valves can become diseased or damaged from a variety of causes. Congenital defects may result in abnormally formed valves. Infections such as rheumatic fever, and bacterial endocarditis can lead to valve damage.

The first prosthetic valvular device was implanted in 1952, and a variety of mechanical, bioprosthetic, and homograft valves are presently in use. Thromboembolic events and sudden structural failure are problems associated with traditional mechanical valves. Bioprosthetic xenograft replacement valves have been developed to reduce the risk of such problemd. Xenograft valves are typically porcine or bovine. However, such valves are limited in their durability, as calcification and fibrotic sheath formation often lead to stenosis and regurgitation, with a 40% reoperation rate 8-10 years after implantation.

SUMMARY OF THE INVENTION

The invention provides an improved replacement cardiac valve. The bioprosthetic heart valve contains an acellular matrix as a structural scaffold and isolated myofibroblasts. The acellular matrix is preferably an acellular homograft, an acellular xenograft, or a synthetic matrix. The matrix is contacted with isolated myofibroblasts, which are allowed to cellularize the matrix. The myofibroblasts are resistant to dedifferentiation during culture prior to implantation and after implantation into a recipient individual. At least 60% of the total collagen produced by the myofibroblasts is type I collagen. Preferably, the

myofibroblasts produce at least 2-fold more type I collagen compared to type III collagen. Reduced type III collagen production is critical to minimizing scar tissue formation in the replacement valve recipient. Accordingly, less than 25%, more preferably less than 20%, and most preferably less than 15% of total collagen production by valve myofibroblasts is type III collagen.

In addition to increased type I collagen production, the myofibroblasts secrete extracellular matrix components, including but not limited to, fibronectin, elastin, and glycosaminoglycans, such as chondroitin sulfate or hyaluronic acid. The myofibroblast cells, are cultured in the presence of factors which inhibit dedifferentiation. The cells are cultured in the presence or absence of an acellular matrix or scaffold. For example, the cells are maintained in an endothelial cell-conditioned media, or grown in the presence of endothelial cells. The two cell types may be in direct contact with one another, e.g., in a coculture, or separated by a membrane which allows diffusion of soluble factors but prevents cell-to-cell contact.

The term "isolated" used in reference to a particular cell type, e.g., a myofibroblast or endothelial cell, means that the cell is substantially free of other cell types or compositions with which it naturally occurs. For example, isolated myofibroblasts are obtained from solid heart leaflet tissue but are separated from other cell types which are present in heart leaflet interstitial tissue. Cells are "isolated" when the particular cell type is at least 60% of a cell population. Preferably, the cells represent at least 75%, more preferably at least 90%, and most preferably at least 99%, of the cell population. Purity is measured by any appropriate standard method, for example, by fluorescence-activated cell sorting (FACS) using cell type-specific markers described herein. A population of cells used to cellularize an acellular valve structure or synthetic structure may be a mixture of two or more different cell types, each of which is isolated. For example, valves are colonized with a mixture of isolated myofibroblasts and isolated endothelial cells.

The myofibroblasts used to cellularize a valve matrix are obtained from a variety of tissue sources, e.g., vascular or dermal tissue. Preferably, the cells are derived from histocompatible (e.g., autologous) mammalian heart leaflet interstitial tissue such as human

heart leaflet interstitial tissue. Alternatively, the cells are derived from other tissue sources, e.g., dermal tissue, and cultured under conditions which promote a myofibroblast-like phenotype.

To inhibit dedifferentiation of myofibroblasts, the cells are maintained in the presence of one or mor growth factors which favor the leaflet myofibroblast phenotype (i.e., contractile and secretory function). The cells are maintained in static culture conditions or subjected to pulsatile flow culture conditions. Growth factors include basic fibroblast growth factor (bFGF). As is discussed above, the cells are cultured in endothelial cell-conditioned media or in physical contact with endothelial cells. Myofibroblasts may be cultured in the presence of a purified or recombinant growth factor. Preferably, the growth factor is derived from an endothelial cell, e.g., purified from endothelial cell conditioned media. The factor is purified using methods known in the art such as standard chromatographic techniques or recombinant cloning technology.

A secretory cell, e.g., one that has been genetically modified to produce a growth factor or matrix component, is used in coculture with isolated myofibroblasts. For example, the secretory cell is of non-endothelial and non-myofibroblast origin.

Myofibroblast cells are cultured under pulsatile flow conditions to enhance production of type I collagen and minimize dedifferentiation. Cellularized valves cultured under such conditions assume the functional anatomy of a native valve. For example, the valve leaflets contain a monolayer of endothelial cells on the external layer and myofibroblasts in the inner layers. The leaflet interstitium contains a non-homogeneous matrix of one or more layers with myofibroblasts present in all layers and with collagen fibrils oriented in more than one direction. The cell culture conditions inhibit apoptosis of a myofibroblast that has been removed from a donor mammal, i.e., a harvested, cultured, transformed or transplanted myofibroblast. The culture method enhances viability and contractile activity of myofibroblasts *in vitro*.

Also within the invention is a genetically-modified myofibroblast. For example, the fibroblast is genetically modified to confer a myofibroblast phenotype, e.g., contractile capability

The modified fibroblast produces increased levels of collagen I (compared to a normal, untreated fibroblast), fibronectin, or

glycosaminoglycans. Genetically-altered cells which have colonized a replacement heart valve are useful as an *in vivo* recombinant protein delivery system to deliver therapeutic polypeptides such as anticoagulant or antithrombotic agents.

A method of manufacturing an artificial heart valve includes the steps of (a) providing an acellular matrix, (b) seeding the matrix with isolated myofibroblasts; and (c) culturing the myofibroblasts under pulsatile flow conditions. Optionally, the matrix is seeded with additional cell types such as endothelial cells. The tissue culture media includes growth and cell signaling factors, e.g., those which are present in endothelial cell-conditioned media. Alternatively, factors are isolated from conditioned media, recombinant, or synthetic.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other embodiments and features of the invention will be apparent form the following description thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

DETAILED DESCRIPTION

The presence of viable functional myofibroblast cells in the cardiac valve is essential for leaflet contractility, production of extracellular matrix, and thus for maintenance of proper valve function. Valves devoid of such cells lack the abilities to grow, repair, and remodel. The replacement valve of the invention a "personal" valve, containing cells derived from histocompatible tissue, such as from the valve recipient patient. The cells dwell within

a non-immunogenic acellular matrix support from a donor. Advantages of such a valve include elimination of need for immune suppression when transplanting cells from a donor and elimination of biocompatibility concerns which accompany the use of biomaterials in tissue engineered valves.

For example, an adult or pediatric valve replacement is made which approximates a normal, unstressed native valve, both in terms of numbers and amounts as well as types of cells and matrix. The valve is fully hemodynamically functional, without need for anticoagulation or immunosuppression, with durability extending potentially to the recipient's natural life-span.

The juvenile sheep chronic implant model of heart valve transplantation is an art recognized model of human transplantation. Ten cryopreserved sheep homograft valves and 5 cryopreserved human xenograft valves were transplanted into the pulmonary position of sheep.. All were evaluated via echocardiography. The data indicate that the homografts were predominantly acellular at 20 weeks. Cell culture of sheep leaflet interstitial cells are cultured as described below and seeded onto valve scaffold. The recellularized valves are evaluated using the sheep model.

Bioprosthetic valve components

A structure which acts as a scaffold is colonized by living cells. The scaffold is obtained from a human homograft cardiac valve (either pulmonary or aortic); a xenograft cardiac valve (e.g., porcine or bovine). Alternatively, it is made from a synthetic polymeric material, (e.g., polylactic / polyglycolic acid). The structure is acellular and has the geometry of a native cardiac valve. In the case of the homograft or xenograft valve, cells are removed from the structure using methods known in the art, e.g., as described in U.S. Patent No. 5,843,182 or WO 96/03093. Acellular human homograft cardiac valves that have been rendered acellular are preferred because of their low antigenicity and similarity to native human valve in geometry and molecular composition. Other compositions such as plastic, metal, or cloth can be used as the valve structure.

Myofibroblasts and Other Cell Types for Cellularization of Valve Structures

The biological valve is decellularized prior to culture with isolated myofibroblasts. The decellularized valve contains other extracellular matrix components such as collagen which confers upon the valve general tensile strength, proteoglycans which absorb stress,

confer flexibility, regulate the extent of collagen fibrils crosslinking, and elastin for leaflet coaptation or valve closure.

Cells e.g., isolated myofibroblasts, are obtained from a donor mammal. Preferably, the mammal is a human, and more preferably, the tissue is obtained by biopsy from the individual to be treated. For example, cells are surgically removed from heart valve tissue, or elsewhere (e.g., dermis). The cells are cultured *ex vivo* to expand the cells. Alternatively, cells are obtained from human cadaver tissue, cultured to expand cell number, and used to cellularize a valve scaffold.

The scaffold is contacted with cells (e.g., myofibroblasts or myofibroblast-like cells, in the presence or absence of endothelial cells). Replacement valves are colonized with cells in manner which resembles a naturally-occurring valve. Naturally-occurring cardiac valve leaflets have three internal layers – ventricularis, spongiosa, and fibrosa. Endothelial cells are present in a single layer around the leaflet's blood-contacting surface. Myofibroblasts are found throughout the 3 layers, with the sparsest population in the fibrosa. Myofibroblasts are aligned with the collagen fibrils in the matrix of the valve. The replacement valve, recellularized as described herein, approximates a naturally-occurring valve, as follows. The ventricularis contains myofibroblasts, multidirectionally oriented collagen, and extensive elastin, which is perpendicular to the free edge. The spongiosa contains myofibroblasts, loosely arranged collagen, and proteoglycans, including chondroitin sulfate and hyaluronic acid. The fibrosa contains fewer myofibroblasts than the other layers, a small number of elastic fibers, and dense collagen that is circumferentially oriented, crimped when relaxed, and elongated under pressure.

Cells incorporated within the valve matrix internal layers are myofibroblasts, i.e., the cells have dual biological function: matrix synthesis and contractility. Myofibroblast phenotype is assessed by immunocytochemistry with the following antibodies: monoclonal anti-alpha-smooth muscle actin, monoclonal anti-vimentin, anti-desmin, monoclonal anti-light chain myosin, monoclonal anti-alpha-tubulin, monoclonal anti-cellular fibronectin, monoclonal anti-chondroitin sulfate (SIGMA, St. Louis, MO), and monoclonal anti-prolyl-4-hydroxylase

The localization and the ability of the cells to synthesize matrix components is assessed by evaluation of mRNA for collagen type I, collagen type III, and elastin. Myofibroblasts are also identified and quantified using

metabolic labeling with collagen amino acids – proline, metabolic labeling with sulfate for GAG analysis, elastin mRNA.

Endothelial cell phenotype is assessed by immunocytochemistry with antibodies to factor VIII (polyclonal antibody, BioGenex) and to CD31(monoclonal antibody, Dako), and by incorporation of DiI-labeled acetylated low density lipoprotein (Biomedical Technologies). Synthesis of proteoglycans is evaluated by radiolabeled sulfate incorporation, and by radiolabeled glucosamine incorporation.

Tissue Culture Conditions

Myofibroblasts are harvested from histocompatible donor tissue, e.g., valve leaflets or dermal tissue, and cultured according to known methods, (e.g., Messier et al., 1994, J. Surg. Res. 57:1-21) or by explant culture. For explant culture, leaflets are scraped to remove endothelium and chopped into 1-3 mm^3 pieces. Pieces are plated in tissue culture flasks or dishes, and myofibroblasts migrate out within 5-7 days. Fibroblasts from vascular, dermal, or other tissue sources are cultured by the same methods. Fibroblasts from these sources acquire the myofibroblast phenotype with the use of dynamic tissue culture conditions and/or cell signaling factors. Culture medium used to grow and maintain myofibroblast cultures is M199, 15% fetal bovine serum, penicillin-streptomycin.

Endothelial cells are cultured according to standard protocols (e.g., Gimbrone et al. ; from femoral vein or artery, jugular vein or artery, or valve leaflet biopsies. Cultures are generated by scraping endothelium, collagenase treatment (0.1%), or explant cultures. Culture medium is the same as for myofibroblast culture. Endothelial cell growth factor (Gibco) is added to promote proliferation. Collagen or gelatin coating of tissue culture dishes or flasks is optionally used to promote cell attachment.

Mixed cultures of myofibroblasts and endothelial cells are cocultured with cell-cell contact. Cells for coculture are generated by either collagenase treatment or explant culture of unscraped leaflets. Cells are separated into isolated single-type populations by flow cytometry using DiI-Ac-LDL to label endothelial cells.

Cells are cultured in a mixed culture without cell-cell contact, but with free diffusion of soluble factors. For example, myofibroblasts and endothelial cells are separated by a cell culture insert composed of a semipermeable membrane, i.e. cellulose acetate. One cell type is grown on a tissue culture dish, while the other grows on the cell culture insert.

Conditioned medium from the co-cultures is collected and separated into fractions by size and by charge using standard protein and proteoglycan column isolation methods. Fractions are analyzed for the ability to promote the myofibroblast phenotype, and for the ability to promote recellularization of valve tissue. Either purified myofibroblast-promoting factor or conditioned media is used to promote growth and dedifferentiation of cells to be used as the cellular component of replacement heart valves.

Cells are typically grown culture prior to seeding for 1-4 weeks. The cells are maintained under standard static tissue culture conditions, in a bioreactor (with or without rotation), or in a pulsatile flow chamber. Cells cultured in a pulsatile flow chamber are either myofibroblasts or mixed-type populations, e.g., a mixture of isolated myofibroblasts and isolated endothelial cells.

Cells which have been genetically modified to produce specific proteins (i.e. myofibroblast differentiation factors, endothelial cell-myofibroblast cell signaling proteins, extracellular matrix components) are cultured as described above. Stem cells (whose phenotype is not permanently determined) are cultured and driven toward a myofibroblast phenotype through incubation with differentiation factors and/or pulsatile culture conditions.

Endothelial cells are isolated by perfusion of vessels or incubation of valve leaflets with collagenase. Culture medium is changed 30 minutes after the initial culture to remove fibroblasts and smooth muscle cells. Cells are cultured in endothelial basal medium 131 with 10% fetal bovine serum and 2ng/ml basic fibroblast growth factor. Endothelial cells are identified by their uptake of DiI-labeled acetylated low density lipoprotein (DiI-Ac-LDL).

Leaflet interstitial cells are the preferred source of myofibroblasts. Leaflet tissue is dissected from the central third of coronary cusps, beginning at the nodule of Arantius and ending at a point 3-5mm from the base. Excised tissue is incubated for 24 h in a 37 degrees C humidified environment with 5% CO_2, 95% air in collagenase solution, then aspirated for thorough cell dispersion. Cells are cultured in tissue culture flasks in M199 medium with 10% FBS.

The ability of interstitial cells or isolated myofibroblast cells to contract is assessed using known methods, e.g., Harris et al. (1980

Cells are cultured on a flexible substrate composed of polydimethyl siloxane. The visible generation of wrinkles on the surface of the rubber reveals cellular contraction.

Methods for seeding the valves include diffusion of cells, dynamic flow conditions, or direct injection. Cells cultured and re-implanted are labeled with a fluorescent tracer prior to implantation, to distinguish between cultured and native cells.

Evaluation of explanted valves

The function and durability of replacement valves is evaluated as follows. Once explanted, valves are placed either into formalin or paraformaldehyde for evaluation at the histological, cellular, and molecular levels, into glutaraldehyde for evaluation by transmission electron microscopy, or into tissue culture medium for specific cellular and molecular assays, tissue culture, or mechanical evaluation.

Histology

Valves are sectioned with a cryostat, a microtome, or an ultramicrotome, depending on the evaluation procedure. Histology stains include hematoxylin and eosin and Miller's elastin stain. Valves are examined for the presence of an intact endothelium and a stroma containing matrix proteins and cells. The presence of elastin, collagen, fibronectin, and chondroitin sulfate proteoglycans is measured.

Transmission electron microscopy (TEM)

Cultured cells and valve sections are examined by TEM for general morphology, cell and matrix types, and cell viability. Cells are examined for the presence of cellular organelles appropriate for contractile and synthetic cell types (i.e. cytoskeletal filaments, endoplasmic reticulum), as well as intercellular communicative junctions.

Mechanical properties

To monitor calcification, calcium content of explanted valves are assessed via the ortho-cresolphthalein complexon method. Evaluation of valve mechanical properties, (e.g., tests for strength, flexibility, low-strain-rate tensile fracture, high-strain-rate extensibility, stress-relaxation, and forced vibration) are carried out using known methods.

Endothelial cell function is assessed by immunocytochemistry for von Willebrand factor, P-selectin, and E-selectin, uptake of ac-LDL, and binding of lectins (i.e. Griffonia simplicifolia) and agglutinin. Markers of contractile properties include smooth muscle actin

and myosin light chain by immunohistochemistry, as well as staining with Texas red-phalloidin, which selectively labels F-actin and has the advantages of stoichiometric binding and negligible non-specific staining. Cytoskeletal markers include vimentin, desmin, and tubulin, by immunohistochemistry. The ability of interstitial cells to synthesize fibronectin and chondroitin sulfate proteoglycans and to modify collagen types I and II is determined by immunohistochemistry and in situ hybridization.

The presence of fibronectin, collagen types I and II, and chondroitin sulfate proteoglycans (core proteins as well as glycosaminoglycan chains) is assessed by immunohistochemistry and Western blotting. Collagen levels are evaluated by hydroxyproline assays known in the art.

Seeding of cells into replacement valve structure

For seeding purposes, harvested primary cells are cultured and used within culture passages 1-5 to preserve phenotype. Myofibroblasts are seeded to populate a valve structure at cell numbers of 10,000-150,000 per ml. The matrix or valve structure is optionally treated prior to cell seeding to promote cell attachment, and during the seeding process to promote migration into internal layers, proliferation and maintenance of valve cell phenotype. Coating compositions include cell signaling factors, growth factors, and extracellular matrix components that were removed from a donor valve tissue during decellularization. Such matrix components are typically not present in the case of a synthetic polymer valve matrix, or may need to be augmented to facilitate seeding. For example, the structure is coated with basic fibroblast growth factor, platelet derived growth factor, endothelial cell growth factor, fibronectin, integrins, collagen type I, chondroitin sulfate, hyaluronic acid, and heparan sulfate. Factors also include cell signaling and differentiation factors isolated from co-cultures of myofibroblasts and endothelial cells, cultured under pulsatile flow conditions.

The valve scaffold matrix is seeded with myofibroblasts first, followed by endothelial cells. Alternatively, the matrix is seeded with myofibroblasts, then incubated with factors to attract endothelial cells *in vivo*, or the matrix is seeded with a mixed population of myofibroblasts and endothelial cells.

Once cells have attached (3-24 hours), the recellularized valve matrix is incubated under pulsatile flow conditions designed to duplicate the cyclic opening and closing under pressure of a native valve. Typical flow values approximate a cardiac output of 2-7.5 liters/min, with a frequency of 60-120 cycles/min and resistances configured to

duplicate back pressures of up to 120 mm Hg for aortic valve and [AMOUNT] mm Hg for pulmonary valve. For example, isolated myofibroblasts (in the presence or absence of endothelials cells) are cultured with a valve structure under normal blood flow conditions. Frequency is 70 bpm with a diastolic pressure of 70 mg Hg and a flow rate of 5 L/min.

Pulsatile flow conditions promote and/or maintain a myofibroblast phenotype. As is discussed above, myofibroblasts are distinguished phenotypically by their content of α-smooth muscle action. Pulsatile flow culture conditions also promote elevated synthesis of Type I collagen by myofibroblasts. Pulsatile flow culture conditions for fibroblasts and myofibroblasts are known in the art, e.g., U.S. Patent No. 5,899,937.

Methods of Enhancing Type I Collagen Production

Myofibroblasts and/or myofibroblast/endothelial mixtures are cultured as described above to increase type I collagen production relative to type III collagen production. The amount of collagen in the valve leaflet is assessed by using known methods, e.g., the 4-hydroxy-proline assay, and also by the Biocolor Sircoll dye assay (Biocolor). The proportions of collagens type I and III in the valve leaflet is an important measure of the health of the tissue. Collagen III is present in scar tissue or healing tissue, and amounts exceeding 15-20% are not appropriate for a functional valve. Types of collagen are evaluated by interrupted gel electrophoresis and by transmission electron microscopy (TEM).

Interrupted gel electrophoresis resolves type I, III, and V collagen, the main subtypes found in leaflets. After radiolabeling with ^{3}H-proline, the tissue is electrophoresed on nonreducing 5% SDS polyacrylamide gel in the presence of 0.05M urea until the dye front has migrated approximately 1/3 of the total run distance. Then 20 microliters of β-mercaptoethanol isadded to each well. Since type III collagen is disulfide bonded, its migration is retarded relative to the α chains of type I and V collagen until the reducing agent is added. Therefore, the α1(III) chain can be resolved from the α1 (I) chain. The α1(V) and α2(V) chains migrate between the α1(I) and theα1(III) chains. The gel is soaked in 10 volumes of sodium salicylate (pH6.0) for 30 min to enhance ^{3}H emission intensity and exposed to Kodak SB X-ray film for fluorography. The ratio of type III to type I collagen will be determined by scanning densitometry. Type I and type III collagen are distinguished as follows: collagen I fibrils are 50-100 nm in diameter, and collagen III fibrils are 25-40 nm (analyzed by TEM). For example, the ratio of collagen is I:III:V = 85:15:5. Orientation of

collagen fibrils varies, e.g., crimped vs. elongated, depending on layer and pressure conditions. Collagen crosslinking is evaluated for extent of crosslinking and type of crosslinking (reducible vs. non-reducible, typical of load-bearing tissues). Quantity of collagen is measured using a standard 4-hydroxyproline assay.

Methods of Inhibiting Apoptosis of Repopulating Cells

The culture conditions and media formulations described herein minimizes apoptosis of primary myofibroblasts. Valves are evaluated for evidence of mitotic activity by the presence of immunoreactivity for proliferating cell nuclear antigen (PCNA), and for evidence of apoptosis by TEM and in situ nick end labeling. Nick end labeling is used to incorporate fluorescent-tagged deoxynucleotides by terminal deoxynucleotidyl transferase, which allows detection of DNA fragmentation that occurs in apoptosis. Morphometric analysis with transmission electron microscopy provides information concerning the status of cellular membranes and organelles at a specific point in time of sampling. Mitotic state and apoptotic state are evaluated to determine the health of the cells.

Characterization of allgraft and homograft valves

The morphology and viability effects of pre-implantation processing on homografts, i.e. cryopreservation or warm ischemia, were assessed using a spectrum of techniques to measure the physiological state of the valve (e.g., examining energetics via NMR, phosphate labeling, and HPLC techniques). The primary valve leaflet interstitial cell was identified as the myofibroblast. Tissue viability requirements for this critical valve cell were determined. The effects of processing and transplantation on apoptosis of valve cells was evaluated.

Sheep pulmonary valve endothelial cells and leaflet interstitial cells are as an animal model. Various media (including media with and without serum) for effects on apoptosis, collagen production, and contractile capabilities. A chronic surgical model (20 week survival) is used optimize animal survival. Four groups of valves are implanted and evaluated for auto-seeding by host cells. Each of the following test groups contains 10 animals per group.

Group 1 - cryopreserved valves

Group 2 - decellularized valves

Group 3 - decellularized valves, pre-incubated in Optimal Serum-Containing Medium Version 1a

Group 4 - decellularized valves, pre-incubated in Optimal Serum-Free Medium Version 1b

Leaflet interstitial cells and endothelial cells are cultured under various conditions, including culture of each cell type separately, co-culture with and without physical contact, and culture in a pulsatile flow environment.

Two groups of valves are implanted and evaluated for auto-seeding by host cells:

Group 5 - decellularized valves, pre-incubated in Optimal Serum-Containing Medium Version 2a

Group 6 - decellularized valves, pre-incubated in Optimal Serum-Free Medium Version 2b

Valves seeded with leaflet interstitial cells alone are tested in short term models with 5 animals per group. Survival times are chosen based on studies of apoptosis in homograft sheep valves. Host endothelialization will be evaluated.

Group 7 – seeded valves, 10-14 day survival

Group 8 - seeded valves, 2 month survival

Group 9 - valves seeded with pulsatile flow, 10-14 day survival

Group 10 - valves seeded with pulsatile flow, 2 month survival

Following short term studies, leaflet interstitial cell-seeded valves are tested in chronic models with 10 animals per group. Host endothelialization is evaluated.

Group 11 - seeded valves

Group 12 - valves seeded with pulsatile flow

To further optimize cell seeding parameters, valves are seeded with leaflet interstitial cells and endothelial cells under various conditions: each cell type separately, co-culture, and culture in a pulsatile flow environment.,

Group 13 – myofibroblasts and endothelial cells cultured separately then seeded, 10-14 day survival

Group 14 – myofibroblasts and endothelial cells cultured separately then seeded, 2 month survival

Group 15 – myofibroblasts and endothelial cells co-cultured then co-seeded, 10-14 day survival

Group 16 – myofibroblasts and endothelial cells co-cultured then co-seeded, 2 month survival

Group 17 – same as group 13 but with pulsatile flow culture conditions

Group 18 – same as group 14 but with pulsatile flow culture conditions

Group 19 – same as group 15 but with pulsatile flow culture conditions

Group 20 – same as group 16 but with pulsatile flow culture conditions

Following short term studies, leaflet interstitial cell-seeded valves are tested in chronic models with 10 animals per group.

Group 21 – myofibroblasts and endothelial cells cultured separately then seeded

Group 22 – myofibroblasts and endothelial cells co-cultured then co-seeded

Group 23 – same as group 21 but with pulsatile flow culture conditions

Group 24 – same as group 22 but with pulsatile flow culture conditions

Data regarding media compostion and culture condition are used to optimize culture of a cellularized replacement valve prior to implantation into a recipient mammal.

Juvenile Sheep Model of for Human Aortic Valve Replacement.

Domestic sheep (Ovis aries): Rambouillet, Dorset, Hampshire, Suffolk Breed mix (30, 6 per valve type) either male or female (20 to 40 weeks of age with body weight 40-50 kg) are commercially available. Prior to implantation, the animals are certified to be free from disease.

The sheep is given Amikacin (10 mg/kg IM) and amoxicillin (5-10 mg/kg IM) and fasted from its daily standardized diet from the evening prior to surgery. On the morning of surgery, the animal is weighed, surgical sites are sheared, and the animal is anesthetized with using standard methods.

The sheep is secured to the operating table in the left side up lateral position. Total volume is maintained at 10 ml/kg body weight of 99% oxygen with a 50-100 m. compensation for dead space at a rate of 12-14 cycles per minute (ABG's checked at 15 minutes - 30 minutes). Left thoracotomy is performed and the chest entered through the fifth intercostal space. A bypass Heparin bonded shunt is inserted from the right atrium to the distal pulmonary artery with a roller pump head in the circuit. The pulmonary artery is mobilized and a vascular clamp is applied just proximal to the bifurcation and below the level of the insertion of the inflow shunt tubing from the roller pump. The native pulmonary valve is excised with the right ventricle being kept empty as a consequence of the right heart bypass circuit as described above. The tissue engineered valve or control is sutured as an interposition graft. The proximal and distal end-to-end anastomoses is accomplished with

running 4-0 Prolene suture. The bypass shunt is occluded and the vascular clamps removed. The homograft or unstented bioprosthesis is oriented with the base closest to the heart to ensure antegrade flow through the graft.

Fresh valves are harvested from one sheep and implanted into another sheep. Valves to be frozen are harvested from an abbatoir as a byproduct of meat packing. They are treated with antibiotics, antifungal agents and cryopreserved. No disease transmission has ever been documented following such treatment. Decellularized valves is carried out using methods known in the art.

Native pulmonary valves are explanted, placed in sterile tissue culture media, and transported in a sealed, autoclaved container to the site of the surgery.

Following implantation and prior to closing, the thoracic cavity is lavaged with warm saline. The fluid from the thoracic cavity is evacuated. The pericardium is closed with Vicryl sutures. The ribs are approximated with Vicryl sutures. The muscle and fascia layers of the chest are approximated with Vicryl sutures (running). The air and blood are evacuated from the thoracic cavity via chest tube. The skin layer of the chest is closed with 2-0 Vicryl subcuticular suture. When the animal is able to breath spontaneously, mechanical ventilation is discontinued and supplemental oxygen substituted.

The animal is placed in the position of sternal recumbency in intensive care. To permit the expulsion of ruminal gas, this position is maintained until the animal regains consciousness. The chest tube is aspirated regularly following the operation and is removed 6 hours after surgery. The animal is awakened with the assistance of a sling and then walked into a pen. Animals receive weekly trans-thoracic echocardiography evaluations. Trans-thoracic Doppler echocardiography will be performed weekly to assess for stenosis or regurgitation. Euthanasia is by overdose of pentabarbitol (360 mg/kg).

Other embodiments are within the following claims.

What is claimed is:

1. A bioprosthetic heart valve comprising an acellular matrix and isolated myofibroblasts wherein least 60% of the total collagen produce by said myofibroblasts is type I collagen.

2. The valve of claim 1, wherein said myofibroblasts produce at least 2-fold greater type I collagen compared to type III collagen.

3. The valve of claim 1, wherein said myofibroblasts produce one or more extracellular matrix components selected from the group consisting of fibronectin, elastin, and glycosaminoglycan.

4. The valve of claim 3, wherein said glycosaminoglycan is chondroitin sulfate or hyaluronic acid.

5. A valve comprising an acellular matrix and an isolated myofibroblast, wherein less than 25% of total collagen production by said myofibroblast is type III collagen.

6. The valve of claim 5, wherein less than 20% of total collagen production by said myofibroblast is type III collagen.

7. The valve of claim 5, wherein less than 15% of total collagen production by said myofibroblast is type III collagen.

8. The valve of claim 5, wherein said myofibroblast is derived from mammalian heart leaflet interstitial tissue.

9. The valve of claim 5, wherein said myofibroblast is derived from vascular or dermal tissue.

10. The valve of claim 5, wherein said myofibroblast is derived from human heart leaflet interstitial tissue.

11. A method of enhancing production of type I collagen by an isolated myofibroblast, comprising culturing said myofibroblast under pulsatile flow conditions

12. The method of claim 11, wherein said myofibroblast is cultured in the presence of basic fibroblast growth factor.

13. The method of claim 11, wherein said myofibroblast is cultured in endothelial cell-conditioned media.

14. The method of claim 11, wherein said myofibrobast is cultured in the presence of an isolated endothelial cell.

15. A method of enhancing viability and contractile activity of myofibroblasts in vitro comprising culturing said myofibroblast under pulsatile flow conditions.

16. The method of claim 15, wherein said myofibroblast is cultured in endothelial cell-conditioned media.

17. The method of claim 15, wherein said myofibrobast is cultured in the presence of an isolated endothelial cell.

18. The method of claim 15, wherein said myofibroblast is cultured in the presence of a purified endothelial cell-derived growth factor, wherein said growth factor inhibit apoptosis of said myofibroblast

19. An isolated myofibroblast, wherein said myofibroblast is genetically altered to increase type I collagen production relative to type III collagen production.

20. A bioprosthetic heart valve comprising the myofibroblast of claim 19.

21. A method of manufacturing an artificial heart valve, comprising:

(a) providing an acellular matrix,

(b) seeding said matrix with isolated myofibroblasts; and

(c) culturing said myofibroblasts under pulsatile flow conditions.

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CARDIAC VALVE REPLACEMENT

The invention provides a replacement heart valve which contains an acellular matrix as a structural scaffold. The scaffold is seeded with isolated myofibroblasts and/or endothelial cells prior to implantation into a recipient mammal.

EXHIBIT D

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BROWN UNIVERSITY

17 February 2000

Ingrid A. Beattie, Ph.D.
Patent Attorney
Fish & Richardson, P.C.
225 Franklin Street
Boston, MA 02110-2804

Dear Ingrid:

Please find enclosed a hand-marked copy of the patent draft. I've tried to incorporate Richard's and my comments as clearly as possible. Let me know if anything is unclear. Our negotiations with Baxter are speeding up, so filing ASAP is even more important now. On that front, the Baxter folks would like the list of prior art that you searched while preparing this application, so they can be prepared to evaluate it as soon as we are able to send it to them. If you can send that list to me by email (just patent #s should be [redacted] helpful.

Comments to accompany the draft:

p. 2 Lines 14-17
The conditioned media will be fractionated by size and charge. The ability of each fraction to influence the myofibroblast phenotype will be assessed as described (i.e. qualitative evaluation by immunocytochemistry and histology for contractile and synthetic capabilities, quantitative evaluation by assays for matrix components including collagen, elastin, glycosaminoglycans). The fraction(s) with highest activity will be purified and sequenced by methods standard in the art.

p. 3 Lines 13-14
We're calling this a cell signaling factor. It may not necessarily influence fibroblasts or myofibroblasts to proliferate more quickly (i.e. a growth factor) but rather, it may influence their phenotype (i.e. secretory, contractile capabilities).

p.3 Lines 30-31
I haven't specified this at this point. We'll certainly be looking at actin and myosin.

p. 5 Line 1
These methods are applicable to matrices other than homografts. Also, the scaffold may be modified to promote ingrowth and avoid regurgitation, and it may be constructed in vitro.

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p. 7 Line 19
Gimbrone MA: Culture of vascular endothelium. Ch. 1 in Spaet T (ed) Progress in hemostasis and thrombosis Vol III. Grune and Stratton Inc. 1976: pp. 1-28.

With regard to including figures, what's your opinion about including just qualitative photos? They would include cultures of myofibroblasts, fluorescent immunocytochemistry results showing expression of cell and matrix markers, and cultures containing endothelial cells mixed in with smooth muscle cells and myofibroblasts. Let me know your thoughts.

Please let me know if you need any other information, and I'll call to be sure you've received this tomorrow.

Sincerely,

Diane Hoffman-Kim, Ph.D.

Attorney's Docket No. 04930-027001

CARDIAC VALVE REPLACEMENT

STATEMENT AS TO FEDERALLY-SPONSORED RESEARCH

BACKGROUND OF THE INVENTION

The invention relates to cardiac valve replacement.

Cardiac valve diseases are prevalent clinical problems, usually requiring prosthetic replacement. Valves can become diseased or damaged from a variety of causes. Congenital defects may result in abnormally formed valves. Infections such as rheumatic fever and bacterial endocarditis can lead to valve damage.

The first prosthetic valvular device was implanted in 1952, and a variety of mechanical, bioprosthetic, and homograft valves are presently in use. Thromboembolic events and sudden structural failure are problems associated with traditional mechanical valves. Bioprosthetic xenograft replacement valves have been developed to reduce the risk of such problems. Xenograft valves are typically porcine or bovine. However, such valves are limited in their durability, as calcification and fibrotic sheath formation often lead to stenosis and regurgitation, with a 40% reoperation rate 8-10 years after implantation. Homograft valve transplants are limited by immune and inflammatory recipient responses, limited donor cell viability, and complex matrix issues resulting in degradation of mechanical performance properties.

SUMMARY OF THE INVENTION

The invention provides an improved replacement cardiac valve. The bioprosthetic heart valve contains an acellular matrix as a structural scaffold and isolated myofibroblasts. The acellular matrix is preferably an acellular homograft, an acellular xenograft, or a synthetic matrix. The matrix is contacted with isolated myofibroblasts, which are allowed to cellularize the matrix. The myofibroblasts are resistant to dedifferentiation during culture prior to implantation and after implantation into a recipient individual. At least 60% of the total collagen produced by the myofibroblasts is type I collagen. Preferably, the

myofibroblasts produce at least 2-fold more type I collagen compared to type III collagen. Reduced type III collagen production is critical to minimizing scar tissue formation in the replacement valve recipient. Accordingly, less than 25%, more preferably less than 20%, and most preferably less than 15% of total collagen production by valve myofibroblasts is type III collagen.

In addition to increased type I collagen production, the myofibroblasts secrete extracellular matrix components, including but not limited to, fibronectin, elastin, and glycosaminoglycans, such as chondroitin sulfate or hyaluronic acid. The myofibroblast cells are cultured in the presence of factors which inhibit dedifferentiation. The cells are cultured in the presence or absence of an acellular matrix or scaffold. For example, the cells are maintained in an endothelial cell-conditioned media, or grown in the presence of endothelial cells. The two cell types may be in direct contact with one another, e.g., in a coculture, or separated by a membrane which allows diffusion of soluble factors but prevents cell-to-cell contact.

The term "isolated" used in reference to a particular cell type, e.g., a myofibroblast or endothelial cell, means that the cell is substantially free of other cell types or compositions with which it naturally occurs. For example, isolated myofibroblasts are obtained from solid heart leaflet tissue but are separated from other cell types which are present in heart leaflet interstitial tissue. Cells are "isolated" when the particular cell type is at least 60% of a cell population. Preferably, the cells represent at least 75%, more preferably at least 90%, and most preferably at least 99%, of the cell population. Purity is measured by any appropriate standard method, for example, by fluorescence-activated cell sorting (FACS) using cell type-specific markers described herein. A population of cells used to cellularize an acellular valve structure or synthetic structure may be a mixture of two or more different cell types, each of which is isolated. For example, valves are colonized with a mixture of isolated myofibroblasts and isolated endothelial cells.

The myofibroblasts used to cellularize a valve matrix are obtained from a variety of tissue sources, e.g., cardiac, vascular or dermal tissue. Preferably, the cells are derived from histocompatible (e.g., autologous) mammalian heart leaflet interstitial tissue such as human

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heart leaflet interstitial tissue. Alternatively, the cells are derived from other tissue sources, e.g., dermal tissue, and cultured under conditions which promote a myofibroblast-like phenotype.

To inhibit dedifferentiation of myofibroblasts, the cells are maintained in the presence of one or more growth factors which favor the leaflet myofibroblast phenotype (i.e., contractile and secretory function). The cells are maintained in static culture conditions or subjected to pulsatile flow culture conditions. Growth factors include basic fibroblast growth factor (bFGF). As is discussed above, the cells are cultured in endothelial cell-conditioned media or in physical contact with endothelial cells. Myofibroblasts may be cultured in the presence of a purified or recombinant growth factor. Preferably, the growth factor is derived from an endothelial cell, e.g., purified from endothelial cell conditioned media. The factor is purified using methods known in the art such as standard chromatographic techniques or recombinant cloning technology.

A secretory cell, e.g., one that has been genetically modified to produce a growth factor or matrix component, is used in coculture with isolated myofibroblasts. For example, the secretory cell is of non-endothelial and non-myofibroblast origin.

Myofibroblast cells are cultured under pulsatile flow conditions to enhance production of type I collagen and minimize dedifferentiation. Cellularized valves cultured under such conditions assume the functional anatomy of a native valve. For example, the valve leaflets contain a monolayer of endothelial cells on the external layer and myofibroblasts in the inner layers. The leaflet interstitium contains a non-homogeneous matrix of one or more layers with myofibroblasts present in all layers and with collagen fibrils oriented in more than one direction. The cell culture conditions inhibit apoptosis of a myofibroblast that has been removed from a donor mammal, i.e., a harvested, cultured, transformed or transplanted myofibroblast. The culture method enhances viability and contractile activity of myofibroblasts *in vitro*.

Also within the invention is a genetically-modified myofibroblast. For example, the fibroblast is genetically modified to confer a myofibroblast phenotype, e.g., contractile capability.

The modified fibroblast produces increased levels of collagen I (compared to a normal, untreated fibroblast), fibronectin, or

glycosaminoglycans. Genetically-altered cells which have colonized a replacement heart valve are useful as an *in vivo* recombinant protein delivery system to deliver therapeutic polypeptides such as anticoagulant or antithrombotic agents, e.g. heparin

A method of manufacturing an artificial heart valve includes the steps of (a) providing an acellular matrix, (b) seeding the matrix with isolated myofibroblasts; and (c) culturing the myofibroblasts under actual or biochemically simulated pulsatile flow conditions. Optionally, the matrix is seeded with additional cell types such as endothelial cells. The tissue culture media includes growth and cell signaling factors, e.g., those which are present in endothelial cell-conditioned media. Alternatively, factors are isolated from conditioned media, recombinant, or synthetic.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other embodiments and features of the invention will be apparent form the following description thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

DETAILED DESCRIPTION

The presence of viable functional myofibroblast cells in the cardiac valve is essential for leaflet contractility, production of extracellular matrix, and thus for maintenance of proper valve function. Valves devoid of such cells lack the abilities to grow, repair, and remodel. The replacement valve of the invention is a "personal" valve, containing cells derived from histocompatible tissue, such as from the valve recipient patient. The cells dwell within or neutered non-antigenic cells

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a non-immunogenic acellular matrix support from a donor. See attached. Advantages of such a valve include elimination of need for immune suppression when transplanting cells from a donor and elimination of biocompatibility concerns which accompany the use of biomaterials in tissue engineered valves.

For example, an adult or pediatric valve replacement is made which approximates a normal, unstressed native valve, both in terms of numbers and amounts as well as types of cells and matrix. The valve is fully hemodynamically functional, without need for anticoagulation or immunosuppression, with durability extending potentially to the recipient's natural life-span.

The juvenile sheep chronic implant model of heart valve transplantation is an art recognized model of human transplantation. Ten cryopreserved sheep homograft valves and 5 cryopreserved human xenograft valves were transplanted into the pulmonary position of sheep.. All were evaluated via echocardiography. The data indicate that the homografts were predominantly acellular at 20 weeks. Cell culture of sheep leaflet interstitial cells are cultured as described below and seeded onto valve scaffold. The recellularized valves are evaluated using the sheep model.

Bioprosthetic valve components

A structure which acts as a scaffold is colonized by living cells. The scaffold is obtained from a human homograft cardiac valve (either pulmonary or aortic); a xenograft cardiac valve (e.g., porcine or bovine). Alternatively, it is made from a synthetic polymeric material, (e.g., polylactic / polyglycolic acid). The structure is acellular and has the geometry of a native cardiac valve. In the case of the homograft or xenograft valve, cells are removed from the structure using methods known in the art, e.g., as described in U.S. Patent No. 5,843,182 or WO 96/03093. Acellular human homograft cardiac valves that have been rendered acellular are preferred because of their low antigenicity and similarity to native human valve in geometry and molecular composition. Other compositions such as plastic, metal, or cloth can be used as the valve structure.

Myofibroblasts and Other Cell Types for Cellularization of Valve Structures

The biological valve is decellularized prior to culture with isolated myofibroblasts. The decellularized valve contains other extracellular matrix components such as collagen which confers upon the valve general tensile strength, proteoglycans which absorb stress,

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confer flexibility, regulate the extent of collagen fibrils crosslinking, and elastin for leaflet coaptation or valve closure.

Cells e.g., isolated myofibroblasts, are obtained from a donor mammal. Preferably, the mammal is a human, and more preferably, the tissue is obtained by biopsy from the individual to be treated. For example, cells are surgically removed from heart valve tissue, or elsewhere (e.g., artery, vein, dermis). The cells are cultured *ex vivo* to expand the cells. Alternatively, cells are obtained from human cadaver tissue, cultured to expand cell number, and used to cellularize a valve scaffold.

The scaffold is contacted with cells (e.g., myofibroblasts or myofibroblast-like cells, in the presence or absence of endothelial cells). Replacement valves are colonized with cells in a manner which resembles a naturally-occurring valve. Naturally-occurring cardiac valve leaflets have three internal layers – ventricularis, spongiosa, and fibrosa. Endothelial cells are present in a single layer around the leaflet's blood-contacting surface. Myofibroblasts are found throughout the 3 layers, with the sparsest population in the fibrosa. Myofibroblasts are aligned with the collagen fibrils in the matrix of the valve. The replacement valve, recellularized as described herein, approximates a naturally-occurring valve, as follows. The ventricularis contains myofibroblasts, multidirectionally oriented collagen, and extensive elastin, which is perpendicular to the free edge. The spongiosa contains myofibroblasts, loosely arranged collagen, and proteoglycans, including chondroitin sulfate and hyaluronic acid. The fibrosa contains fewer myofibroblasts than the other layers, a small number of elastic fibers, and dense collagen that is circumferentially oriented, crimped when relaxed, and elongated under pressure.

Too stringent? We prefer p.3, Lines 20-24

Cells incorporated within the valve matrix internal layers are myofibroblasts, i.e., the cells have dual biological function: matrix synthesis and contractility. Myofibroblast phenotype is assessed by immunocytochemistry with the following antibodies: monoclonal anti-alpha-smooth muscle actin, monoclonal anti-vimentin, anti-desmin, monoclonal anti-light chain myosin, monoclonal anti-alpha-tubulin, monoclonal anti-cellular fibronectin, monoclonal anti-chondroitin sulfate (SIGMA, St. Louis, MO), and monoclonal anti-prolyl-4-hydroxylase (Dako Carpinteria, CA). The localization and the ability of the cells to synthesize matrix components are assessed by evaluation of mRNA for collagen type I, collagen type III, and elastin, ~~Myofibroblasts are also identified and quantified using~~ standard histological methods (Movat's pentachrome stain) and by incorporation of proline for collagen and sulfate or glucosamine for proteoglycans.

metabolic labeling with collagen amino acids – proline, metabolic labeling with sulfate for GAG analysis, elastin mRNA.

Endothelial cell phenotype is assessed by immunocytochemistry with antibodies to factor VIII (polyclonal antibody, BioGenex, San Ramon CA) and to CD31 (monoclonal antibody, Dako, Carpinteria CA), and by incorporation of DiI-labeled acetylated low density lipoprotein (Biomedical Technologies, Stoughton MA). Synthesis of proteoglycans is evaluated by radiolabeled sulfate incorporation, and by radiolabeled glucosamine incorporation.

Tissue Culture Conditions

Myofibroblasts are harvested from histocompatible donor tissue, e.g., valve leaflets or dermal tissue, and cultured according to known methods, (e.g., Messier et al., 1994, J. Surg. Res. 57:1-21) or by explant culture. For explant culture, leaflets are scraped to remove endothelium and chopped into 1-3 mm^3 pieces. Pieces are plated in tissue culture flasks or dishes, and myofibroblasts migrate out within 5-7 days. Fibroblasts from vascular, dermal, or other tissue sources are cultured by the same methods. Fibroblasts from these sources acquire the myofibroblast phenotype with the use of dynamic tissue culture conditions and/or cell signaling factors. Culture medium used to grow and maintain myofibroblast cultures is M199, 5-15% fetal bovine serum, penicillin-streptomycin.

Endothelial cells are cultured according to standard protocols (e.g., Gimbrone et al., J. Cell. Biol. 49:) from femoral vein or artery, jugular vein, or radial artery, or valve leaflet biopsies. Cultures are generated by scraping endothelium, collagenase treatment (0.1%), or explant cultures. Culture medium is the same as for myofibroblast culture, or may be serum free with Endothelial cell growth factor (Gibco, Gaithersburg MD) added to promote proliferation. Collagen or gelatin coating of tissue culture dishes or flasks is optionally used to promote cell attachment.

Mixed cultures of myofibroblasts and endothelial cells are cocultured with cell-cell contact. Cells for coculture are generated by either collagenase treatment or explant culture of unscraped leaflets. Cells are separated into isolated single-type populations by flow cytometry using DiI-Ac-LDL to label endothelial cells.

Cells are cultured in a mixed culture without cell-cell contact, but with free diffusion of soluble factors. For example, myofibroblasts and endothelial cells are separated by a cell culture insert composed of a semipermeable membrane, i.e. cellulose acetate. One cell type is grown on a tissue culture dish, while the other grows on the cell culture insert. Alternatively, the two cell types are grown on opposite sides of a semipermeable membrane.

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Conditioned medium from the co-cultures is collected and separated into fractions by size and by charge using standard protein and proteoglycan column isolation methods. Fractions are analyzed for the ability to promote the myofibroblast phenotype, and for the ability to promote recellularization of valve tissue. Either purified myofibroblast-promoting factor or conditioned media is used to promote growth and dedifferentiation of cells to be used as the cellular component of replacement heart valves.

Cells are typically grown in culture prior to seeding for 1-4 weeks. The cells are maintained under standard static tissue culture conditions, in a bioreactor (with or without rotation), or in a pulsatile flow chamber. Cells cultured in a pulsatile flow chamber are either myofibroblasts or mixed-type populations, e.g., a mixture of isolated myofibroblasts and isolated endothelial cells.

Cells which have been genetically modified to produce specific proteins (i.e. myofibroblast differentiation factors, endothelial cell-myofibroblast cell signaling proteins, extracellular matrix components) are cultured as described above. Stem cells (whose phenotype is not permanently determined) are cultured and driven toward a myofibroblast phenotype through incubation with differentiation factors and/or pulsatile culture conditions.

Endothelial cells are isolated by perfusion of vessels or incubation of valve leaflets with collagenase. Culture medium is changed 30 minutes after the initial culture to remove fibroblasts and smooth muscle cells. Cells are cultured in endothelial basal medium 131 with 10% fetal bovine serum and 2ng/ml basic fibroblast growth factor. Endothelial cells are identified by their uptake of DiI-labeled acetylated low density lipoprotein (DiI-Ac-LDL).

Leaflet interstitial cells are the preferred source of myofibroblasts. Leaflet tissue is dissected from the central third of coronary cusps, beginning at the nodule of Arantius and ending at a point 3-5mm from the base. Excised tissue is incubated for 24 h in a 37 degrees C humidified environment with 5% CO_2, 95% air in collagenase solution, then aspirated for thorough cell dispersion. Cells are cultured in tissue culture flasks in M199 medium with 10% FBS.

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The ability of interstitial cells or isolated myofibroblast cells to contract is assessed using known methods, e.g., Harris et al. (1980(Science 208:177, 1980
Cells are cultured on a flexible substrate composed of polydimethyl siloxane. The visible generation of wrinkles on the surface of the rubber reveals cellular contraction.

Methods for seeding the valves include diffusion of cells, dynamic flow conditions, or direct injection. Cells cultured and re-implanted are labeled with a fluorescent tracer prior to implantation, to distinguish between cultured and native cells.

Evaluation of explanted valves

The function and durability of replacement valves is evaluated as follows. Once explanted, valves are placed either into formalin or paraformaldehyde for evaluation at the histological, cellular, and molecular levels, into glutaraldehyde for evaluation by transmission electron microscopy, or into tissue culture medium for specific cellular and molecular assays, tissue culture, or mechanical evaluation.

Histology

Valves are sectioned with a cryostat, a microtome, or an ultramicrotome, depending on the evaluation procedure. Histological stains include hematoxylin and eosin, ~~and~~ Miller's elastin stain, Movat's pentachrome stain, and von Kossa stain. Valves are examined for the presence of an intact endothelium and a stroma containing matrix proteins and cells. The presence of elastin, collagen, fibronectin, and ~~chondroitin sulfate proteoglycans~~ glycosaminoglycans is ~~measured~~ assessed.

Transmission electron microscopy (TEM)

Cultured cells and valve sections are examined by TEM for general morphology, cell and matrix types, and cell viability. Cells are examined for the presence of cellular organelles appropriate for contractile and synthetic cell types (i.e. cytoskeletal filaments, endoplasmic reticulum), as well as intercellular communicative junctions.

Mechanical properties

To monitor calcification, calcium content of explanted valves are assessed via ~~the ortho-cresolphthalein complexon method~~ atomic absorption spectroscopy. Evaluation of valve mechanical properties, (e.g., tests for strength, flexibility, low-strain-rate tensile fracture, high-strain-rate extensibility, stress-relaxation, and forced vibration) are carried out using known methods.

Endothelial cell function is assessed by immunocytochemistry for von Willebrand factor, ~~P-selectin, and E-selectin~~ and CD31, and by uptake of ac-LDL, ~~and binding of lectins (i.e. Griffonia simplicifolia) and agglutinin~~. Markers of contractile properties include smooth muscle actin

and myosin light chain by immunohistochemistry, as well as staining with Texas red-phalloidin, which selectively labels F-actin and has the advantages of stoichiometric binding and negligible non-specific staining. Cytoskeletal markers include vimentin, desmin, and tubulin, by immunohistochemistry. The ability of ~~interstitial cells~~ myofibroblasts to synthesize fibronectin and chondroitin sulfate proteoglycans and to modify collagen types I and ~~II~~ III is determined by immunohistochemistry and in situ hybridization.

The presence of fibronectin, collagen types I and ~~II~~ III, and chondroitin sulfate proteoglycans (core proteins as well as glycosaminoglycan chains) is assessed by immunohistochemistry and Western blotting. Collagen levels are evaluated by hydroxyproline assays known in the art, as well as with the Sircol dye assay (Accurate Scientific, Westbury NJ)

Seeding of cells into replacement valve structure

For seeding purposes, harvested primary cells are cultured and used within culture passages 1-5 to preserve phenotype. Myofibroblasts are seeded to populate a valve structure at cell numbers of 10,000-150,000 per ml. The matrix or valve structure is optionally treated prior to cell seeding to promote cell attachment, and during the seeding process to promote migration into internal layers, proliferation and maintenance of valve cell phenotype. Coating compositions include cell signaling factors, growth factors, and extracellular matrix components that were removed from a donor valve tissue during decellularization. Such matrix components are typically not present in the case of a synthetic polymer valve matrix, or may need to be augmented to facilitate seeding. For example, the structure is coated with basic fibroblast growth factor, platelet derived growth factor, endothelial cell growth factor, fibronectin, integrins, collagen type I, chondroitin sulfate, hyaluronic acid, and heparan sulfate. Factors also include cell signaling and differentiation factors isolated from co-cultures of myofibroblasts and endothelial cells, cultured under pulsatile flow conditions.

The valve scaffold matrix is seeded with myofibroblasts first, followed by endothelial cells. Alternatively, the matrix is seeded with myofibroblasts, then incubated with factors to attract endothelial cells *in vivo*, or the matrix is seeded with a mixed population of myofibroblasts and endothelial cells. Alternatively, the matrix is modified first mechanically and/~~or~~ or biochemically ~~Incubated with factors 1st~~ before adding cells)

Once cells have attached (3-24 hours), the recellularized valve matrix is incubated under pulsatile flow conditions designed to duplicate the cyclic opening and closing under pressure of a native valve. Typical flow values approximate a cardiac output of 2-7.5 liters/min, with a frequency of 60-120 cycles/min and resistances configured to

Unique packaging and attractant vehicles may be required to retain such ~~and~~ factors in contact with cells during cell growth, migration, and differentiation.

Alternatively, signaling proteins produced in response to energy dissipation, which regulate increased production of α-smooth muscle actin and related contractile compounds, can be introduced directly or via a secretory cell to drive myofibroblast phenotype.

directly insert factor in matrix or secretory cell

or introduce factor by cells gene therapy according to standard methods.

duplicate back pressures of up to 120 mm Hg for aortic valve, ~~pulmonary valve~~. For example, isolated myofibroblasts (in the presence or absence of endothelials cells) are cultured with a valve structure under normal blood flow conditions. Frequency is 70 bpm with a diastolic pressure of 70 mg Hg and a flow rate of 5 L/min.

Pulsatile flow conditions promote and/or maintain a myofibroblast phenotype. As is discussed above, myofibroblasts are distinguished phenotypically by their content of α-smooth muscle actin. Pulsatile flow culture conditions also promote elevated synthesis of Type I collagen by myofibroblasts. Pulsatile flow culture conditions for fibroblasts and myofibroblasts are known in the art, e.g., U.S. Patent No. 5,899,937.

Methods of Enhancing Type I Collagen Production

Myofibroblasts and/or myofibroblast/endothelial mixtures are cultured as described above to increase type I collagen production relative to type III collagen production. The amount of collagen in the valve leaflet is assessed by using known methods, e.g., the 4-hydroxy-proline assay, and also by the Biocolor Sircol dye assay (Biocolor). The proportions of collagens type I and III in the valve leaflet is an important measure of the health of the tissue. Collagen III is present in scar tissue or healing tissue, and amounts exceeding 15-20% are not appropriate for a functional valve. Types of collagen are evaluated by interrupted gel electrophoresis and by transmission electron microscopy (TEM).

Accurate Scientific, Westbury, NJ

Interrupted gel electrophoresis resolves type I, III, and V collagen, the main subtypes found in leaflets. After radiolabeling with ^{3}H-proline, the tissue is electrophoresed on nonreducing 5% SDS polyacrylamide gel in the presence of 0.05M urea until the dye front has migrated approximately 1/3 of the total run distance. Then 20 microliters of β-mercaptoethanol isadded to each well. Since type III collagen is disulfide bonded, its migration is retarded relative to the α chains of type I and V collagen until the reducing agent is added. Therefore, the α1(III) chain can be resolved from the α1 (I) chain. The α1(V) and α2(V) chains migrate between the α1(I) and theα1(III) chains. The gel is soaked in 10 volumes of sodium salicylate (pH6.0) for 30 min to enhance ^{3}H emission intensity and exposed to Kodak SB X-ray film for fluorography. The ratio of type III to type I collagen will be determined by scanning densitometry. Type I and type III collagen are distinguished as follows: collagen I fibrils are 50-100 nm in diameter, and collagen III fibrils are 25-40 nm (analyzed by TEM). For example, the ratio of collagen is I:III:V = 85:15:5. Orientation of

collagen fibrils varies, e.g., crimped vs. elongated, depending on layer and pressure conditions. Collagen crosslinking is evaluated for extent of crosslinking and type of crosslinking (reducible vs. non-reducible, typical of load-bearing tissues). Quantity of collagen is measured using a standard 4-hydroxyproline assay.

Methods of Inhibiting Apoptosis of Repopulating Cells

The culture conditions and media formulations described herein minimize apoptosis of primary myofibroblasts. Valves are evaluated for evidence of mitotic activity by the presence of immunoreactivity for proliferating cell nuclear antigen (PCNA), and for evidence of apoptosis by TEM and in situ nick end labeling. Nick end labeling is used to incorporate fluorescent-tagged deoxynucleotides by terminal deoxynucleotidyl transferase, which allows detection of DNA fragmentation that occurs in apoptosis. Morphometric analysis with transmission electron microscopy provides information concerning the status of cellular membranes and organelles at a specific point in time of sampling. Mitotic state and apoptotic state are evaluated to determine the health of the cells.

Characterization of allograft and homograft valves

The morphology and viability effects of pre-implantation processing on homografts, i.e. cryopreservation or warm ischemia, were assessed using a spectrum of techniques to measure the physiological state of the valve (e.g., examining energetics via NMR, phosphate labeling, and HPLC techniques). The primary valve leaflet interstitial cell was identified as the myofibroblast. Tissue viability requirements for this critical valve cell were determined. The effects of processing and transplantation on apoptosis of valve cells was evaluated.

Sheep pulmonary valve endothelial cells and leaflet interstitial cells are as an animal model. Various media (including media with and without serum) for effects on apoptosis, collagen production, and contractile capabilities. A chronic surgical model (20 week survival) is used to optimize animal survival. Four groups of valves are implanted and evaluated for auto-seeding by host cells. Each of the following test groups contains 10 animals per group.

Group 1 - cryopreserved valves

Group 2 - decellularized valves

Group 3 - decellularized valves, pre-incubated in Optimal Serum-Containing Medium Version 1a

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Group 4 - decellularized valves, pre-incubated in Optimal Serum-Free Medium Version 1b

Leaflet interstitial cells and endothelial cells are cultured under various conditions, including culture of each cell type separately, co-culture with and without physical contact, and culture in a pulsatile flow environment.

Two groups of valves are implanted and evaluated for auto-seeding by host cells:

Group 5 - decellularized valves, pre-incubated in Optimal Serum-Containing Medium Version 2a

Group 6 - decellularized valves, pre-incubated in Optimal Serum-Free Medium Version 2b

Valves seeded with leaflet interstitial cells alone are tested in short term models with 5 animals per group. Survival times are chosen based on studies of apoptosis in homograft sheep valves. Host endothelialization will be evaluated.

Group 7 – seeded valves, 10-14 day survival

Group 8 - seeded valves, 2 month survival

Group 9 - valves seeded with pulsatile flow, 10-14 day survival

Group 10 - valves seeded with pulsatile flow, 2 month survival

Following short term studies, leaflet interstitial cell-seeded valves are tested in chronic models with 10 animals per group. Host endothelialization is evaluated.

Group 11 - seeded valves

Group 12 - valves seeded with pulsatile flow

To further optimize cell seeding parameters, valves are seeded with leaflet interstitial cells and endothelial cells under various conditions: each cell type separately, co-culture, and culture in a pulsatile flow environment.,

Group 13 – myofibroblasts and endothelial cells cultured separately then seeded, 10-14 day survival

Group 14 – myofibroblasts and endothelial cells cultured separately then seeded, 2 month survival

Group 15 – myofibroblasts and endothelial cells co-cultured then co-seeded, 10-14 day survival

Group 16 – myofibroblasts and endothelial cells co-cultured then co-seeded, 2 month survival

Group 17 – same as group 13 but with pulsatile flow culture conditions

Group 18 – same as group 14 but with pulsatile flow culture conditions

Group 19 – same as group 15 but with pulsatile flow culture conditions

Group 20 – same as group 16 but with pulsatile flow culture conditions

Following short term studies, leaflet interstitial cell-seeded valves are tested in chronic models with 10 animals per group.

Group 21 – myofibroblasts and endothelial cells cultured separately then seeded

Group 22 – myofibroblasts and endothelial cells co-cultured then co-seeded

Group 23 – same as group 21 but with pulsatile flow culture conditions

Group 24 – same as group 22 but with pulsatile flow culture conditions

Data regarding media compostion and culture condition are used to optimize culture of a cellularized replacement valve prior to implantation into a recipient mammal.

Juvenile Sheep Model of for Human Aortic Valve Replacement.

Domestic sheep (Ovis aries): Rambouillet, Dorset, Hampshire, Suffolk Breed mix (30, 6 per valve type) either male or female (20 to 40 weeks of age with body weight 40-50 kg) are commercially available. Prior to implantation, the animals are certified to be free from disease.

The sheep is given Amikacin (10 mg/kg IM) and amoxicillin (5-10 mg/kg IM) and fasted from its daily standardized diet from the evening prior to surgery. On the morning of surgery, the animal is weighed, surgical sites are sheared, and the animal is anesthetized with using standard methods.

The sheep is secured to the operating table in the left side up lateral position. Total volume is maintained at 10 ml/kg body weight of 99% oxygen with a 50-100 m. compensation for dead space at a rate of 12-14 cycles per minute (ABG's checked at 15 minutes - 30 minutes). Left thoracotomy is performed and the chest entered through the fifth intercostal space. A bypass Heparin bonded shunt is inserted from the right atrium to the distal pulmonary artery with a roller pump head in the circuit. The pulmonary artery is mobilized and a vascular clamp is applied just proximal to the bifurcation and below the level of the insertion of the inflow shunt tubing from the roller pump. The native pulmonary valve is excised with the right ventricle being kept empty as a consequence of the right heart bypass circuit as described above. The tissue engineered valve or control is sutured as an interposition graft. The proximal and distal end-to-end anastomoses is accomplished with

running 4-0 Prolene suture. The bypass shunt is occluded and the vascular clamps removed. The homograft or unstented bioprosthesis is oriented with the base closest to the heart to ensure antegrade flow through the graft.

Fresh valves are harvested from one sheep and implanted into another sheep. Valves to be frozen are harvested from an abbatoir as a byproduct of meat packing. They are treated with antibiotics, antifungal agents and cryopreserved. No disease transmission has ever been documented following such treatment. Decellularized valves is carried out using methods known in the art.

Native pulmonary valves are explanted, placed in sterile tissue culture media, and transported in a sealed, autoclaved container to the site of the surgery.

Following implantation and prior to closing, the thoracic cavity is lavaged with warm saline. The fluid from the thoracic cavity is evacuated. The pericardium is closed with Vicryl sutures. The ribs are approximated with Vicryl sutures. The muscle and fascia layers of the chest are approximated with Vicryl sutures (running). The air and blood are evacuated from the thoracic cavity via chest tube. The skin layer of the chest is closed with 2-0 Vicryl subcuticular suture. When the animal is able to breath spontaneously, mechanical ventilation is discontinued and supplemental oxygen substituted.

The animal is placed in the position of sternal recumbency in intensive care. To permit the expulsion of ruminal gas, this position is maintained until the animal regains consciousness. The chest tube is aspirated regularly following the operation and is removed 6 hours after surgery. The animal is awakened with the assistance of a sling and then walked into a pen. Animals receive weekly trans-thoracic echocardiography evaluations. Trans-thoracic Doppler echocardiography will be performed weekly to assess for stenosis or regurgitation. Euthanasia is by overdose of pentabarbitol (360 mg/kg).

Other embodiments are within the following claims.

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What is claimed is:

1. A bioprosthetic heart valve comprising an acellular matrix and isolated myofibroblasts wherein at least 60% of the total collagen produced by said myofibroblasts is type I collagen.

2. The valve of claim 1, wherein said myofibroblasts produce at least 2-fold greater type I collagen compared to type III collagen.

3. The valve of claim 1, wherein said myofibroblasts produce one or more extracellular matrix components selected from the group consisting of fibronectin, elastin, and glycosaminoglycan.

4. The valve of claim 3, wherein said glycosaminoglycan is chondroitin sulfate or hyaluronic acid.

5. A valve comprising an acellular matrix and an isolated myofibroblast, wherein less than 25% of total collagen production by said myofibroblast is type III collagen.

6. The valve of claim 5, wherein less than 20% of total collagen production by said myofibroblast is type III collagen.

7. The valve of claim 5, wherein less than 15% of total collagen production by said myofibroblast is type III collagen.

8. The valve of claim 5, wherein said myofibroblast is derived from mammalian heart leaflet interstitial tissue.

mammalian or human

9. The valve of claim 5, wherein said myofibroblast is derived from vascular or dermal tissue.

10. The valve of claim 5, wherein said myofibroblast is derived from human heart leaflet interstitial tissue.

Also include - a valve that from a mechanical, engineering standpoint, approximates a native human valve. (i.e. Strength, Stress/strain, etc.)

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11. A method of enhancing production of type I collagen by an isolated myofibroblast, comprising culturing said myofibroblast under pulsatile flow conditions

12. The method of claim 11, wherein said myofibroblast is cultured in the presence of basic fibroblast growth factor.

13. The method of claim 11, wherein said myofibroblast is cultured in endothelial cell-conditioned media.

14. The method of claim 11, wherein said myofibrobast is cultured in the presence of an isolated endothelial cell.

15. A method of enhancing viability and contractile activity of myofibroblasts in vitro comprising culturing said myofibroblast under pulsatile flow conditions.

16. The method of claim 15, wherein said myofibroblast is cultured in endothelial cell-conditioned media.

17. The method of claim 15, wherein said myofibrobast is cultured in the presence of an isolated endothelial cell.

18. The method of claim 15, wherein said myofibroblast is cultured in the presence of a purified endothelial cell-derived growth factor, wherein said growth factor inhibits apoptosis of said myofibroblast

19. An isolated myofibroblast, wherein said myofibroblast is genetically altered to increase type I collagen production relative to type III collagen production.

20. A bioprosthetic heart valve comprising the myofibroblast of claim 19.

21. A method of manufacturing an artificial heart valve, comprising:

(a) providing an acellular matrix,

(b) seeding said matrix with isolated myofibroblasts; and

(c) culturing said myofibroblasts under biochemically actual or simulated pulsatile flow conditions.

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CARDIAC VALVE REPLACEMENT

The invention provides a replacement heart valve which contains an acellular matrix as a structural scaffold. The scaffold is seeded with isolated myofibroblasts and/or endothelial cells prior to implantation into a recipient mammal.

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